CERTIFICATE OF MAIL[redacted]BY "EXPRESS MAIL"
I hereby certify that this c[redacted]ndence is being deposited with the US Postal Service[redacted]ess Mail Post Office to Addressee" service under 37 CFR 1.10, [redacted]press Mail Label No. EM096172282US, and addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231 on the date shown below:

Date: June 23, 1997 By: Avis Ridenour
Avis Ridenour

**PATENT**
**Docket No. GC372**

# IMPROVED *ENTEROBACTERIACEAE* FERMENTATION STRAINS

## FIELD OF THE INVENTION

The present invention generally relates to improved fermentation strains of the family *Enterobacteriaceae* and specifically to methods for modifying phenotypic characteristics of the strains relevant to growth conditions. In particular, the present invention relates to the identification of a cryptic plasmid found within a strain of *Enterobacteriaceae* which modulates mobilization properties of other resident plasmids while providing advantageous growth characteristics.

## BACKGROUND OF THE INVENTION

There are numerous commercially important compounds in the carbohydrate pathway of bacterial strains of the family *Enterobacteriaceae* including among others 2-KLG, a precursor to ascorbic acid; idonic acid; L-gluconic acid; and 2,5-DKG. Biocatalyic processes have been developed for the production of these compounds. In particular, Anderson et al., (1985, Science 230:144-149) disclose a metabolic pathway for *Erwinia herbicola* that permits the bioconversion of D-glucose to 2-KLG in a single fermentative step. In this bioconversion, there are a variety of intermediates and one step involves the reduction of 2,5-DKG to 2-KLG which is catalyzed by a recombinantly introduced NADPH-dependent 2,5-DKG reductase. In large scale fermentation of *Enterobacteriaceae* strains containing recombinantly introduced proteins, there remain concerns of a regulatory nature associated with the recombinant nature of the organism.

Vladimir Dellic discloses a summary of a study of organisms for conversion of glucose to ketoacids on internet address "svibor@znanost.hr". This summary discloses the presence in *Erwinia citreus* ATCC accession number 31623 of a plasmid of 3.8 kb, designated pPZG500. Dellic discloses the introduction of a tetracycline resistance gene into pPZG500 which was used to clone a 2,5-DKG reductase gene.

There remains a need to develop improved *Enterobacteriaceae* fermentation strains that have desirable phenotypic characteristics relative to growth conditions and which minimize or eliminate the mobilization properties of resident plasmids under fermentation conditions.

## SUMMARY OF THE INVENTION

The present invention generally relates to improved bacterial fermentation strains of the family *Enterobacteriaceae*. Specifically, the present invention relates to the identification and isolation of a 3.8 kb cryptic plasmid found in strains of *Enterobacteriaceae*. In a preferred embodiment, the *Enterobacteriaceae* strain is a recombinant strain.

The present invention is based in part upon the unexpected discovery that elimination of the cryptic plasmid from the *Enterobacteriaceae* strain *Pantoea* allows for growth of the organism at higher temperatures, thereby decreasing the time for production of desired compounds in the carbohydrate pathway. This discovery has the commercial benefit of potentially reducing both the capital cost and starting materials cost of large scale *Enterobacteriaceae* biocatalysis used in the production of desirable end-products, such as 2-keto-L-gluconic acid, a precursor of ascorbic acid.

The present invention is also based in part on the unexpected discovery that elimination of the cryptic plasmid from *Pantoea* reduces the mobilization properties of *Pantoea* resident plasmids thereby creating a safer and more desirable fermentation strain for the production of materials ultimately intended for human consumption, such as ascorbic acid.

The present invention provides a method for preparing an improved *Enterobacteriaceae* strain from a progenitor *Enterobacteriaceae* strain containing a cryptic plasmid, comprising the step of eliminating the cryptic plasmid from the progenitor strain thereby creating the improved strain. Preferably, the nucleic acid sequence of a 3.8 kb cryptic plasmid according to the invention comprises the plasmid designated herein as pS. The nucleic acid sequence of pS is provided herein and provides a means for identifying plasmids according to the invention which exist in *Enterobacteriaceae* species. The present invention also provides a method for reducing the mobilization properties of plasmids residing within a progenitor *Enterobacteriaceae* strain containing a cryptic plasmid comprising the step of eliminating part or all of the cryptic plasmid from the strain. In an alternative embodiment, the cryptic plasmid nucleic acid is mutated via recombinant DNA techniques to reduce the mobilization properties and/or produce the desirable growth characteristics.

In another embodiment, the present invention provides for an isolated nucleic acid having the sequence as shown in SEQ ID NO:1 and NO:2. In another embodiment, the present invention provides for an isolated amino acid as shown in SEQ ID NO:3. In yet another embodiment, the present invention provides a recombinant host cell produced by methods of the present invention. In one embodiment of the present invention, the host cell

is an *Enterobacteriaceae* strain and in another embodiment, the host cell is *Pantoea citrea* having ATCC accession number 31940.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1F illustrates the nucleic acid sequence of cryptic plasmid designated herein as pS. The nucleic acid sequence is illustrated in two halves, SEQ ID NO: 1, Figures 1A-1C and SEQ ID NO: 2 Figures 1D-1F. The amino acid sequence corresponding to the largest open reading frame (SEQ ID NO:3) is translated in Figures 1A-1C.

Figure 2 illustrates the growth at 28°C, 32°C, and 36°C in ML5 media of a strain containing the cryptic plasmid (pS+) vs a strain that has been cured of the cryptic plasmid (pS-).

Figure 3 illustrates the growth at 28°C, 32°C, and 36°C in Luria broth of a strain containing the cryptic plasmid (pS+) vs a strain that has been cured of the cryptic plasmid (pS-).

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

### Definitions

As used herein, the family "*Enterobacteriaceae* " refers to fermentative bacterial strains having the general characteristics of being gram negative, oxidase-negative and being facultatively anaerobic. Preferred *Enterobacteriaceae* strains are those that are able to produce 2,5-diketo-D-gluconic acid from D-glucose solutions. Included in the family of *Enterobacteriaceae* which are able to produce 2,5-diketo-D-gluconic acid from D-glucose solutions are the genus *Erwinia*, *Enterobacter*, *Gluconobacter* and *Pantoea,* for example. Compounds of interest in the microbial carbohydrate pathway, include but are not limited to D-gluconate (GA), 2-keto-D-gluconate (2KDG), 2,5-diketo-D-gluconate (2,5DKG), 5DKG, 2-keto-L-gluconic acid (2KLG), L-idonic acid (IA) and ascorbic acid. In the present invention, a preferred *Enterobacteriaceae* fermentation strain is *Pantoea citrea* and preferred end compounds include idonic acid, 2KLG and ascorbic acid.

It is well understood in the art that the acidic derivatives of saccharides, may exist in a variety of ionization states depending upon their surrounding media, if in solution, or out of solution from which they are prepared if in solid form. The use of a term, such as, for example, gluconic acid, to designate such molecules is intended to include all ionization states of the organic molecule referred to. Thus, for example, both "D-gluconic acid" and "D-gluconate" refer to the same organic moiety, and are not intended to specify particular

ionization states. The present invention encompasses the unionized forms of derivatives of saccharides, such as, for example, the sodium, potassium or other salt.

As used herein, the phrase "progenitor strain" refers to an *Enterobacteriaceae* strain containing a cryptic plasmid. The term "cryptic plasmid" refers to a plasmid found naturally occurring in a *Enterobacteriaceae* strain which when deleted from the progenitor strain alters the phenotypic growth characteristics or alters mobilization properties of other *Enterobacteriaceae* resident plasmids.

A preferred cryptic plasmid is designated herein as "pS" and refers to a 3.8 kb nucleic acid having the sequence as depicted in SEQ ID NO:1 and SEQ ID NO:2. However, the present invention further encompasses homologs and variations of SEQ ID NO:1 and SEQ ID NO:2 that retain at least one functional characteristic associated with SEQ ID NO:1 and NO:2, i.e., improved phenotypic growth characteristics or reduction of resident plasmid mobilization properties. Due to the degeneracy of the genetic code, a variety of nucleic acids could encode a deduced amino acid sequence encoded by an open reading frame present in SEQ ID NO: 1 and SEQ ID NO:2. Cryptic plasmids according to the present invention encompasse all such nucleic acid variations.

Preferred nucleic acid homologs or variations are those having at least 80%, at least 90% and at least 95% identity to SEQ ID NO: 1 and SEQ ID NO:2. Preferred nucleic acid homologs or variations hybridize under high stringency conditions. The deduced amino acid sequence (SEQ ID NO:3) encoded by the open reading frame shown in SEQ ID NO:1 is illustrated in Figures 1A-1C.

As used herein, the term "recombinant" refers to an *Enterobacteriaceae* strain that contains nucleic acid not naturally occurring in the strain which has been introduced into the strain using recombinant techniques.

As used herein, the term "improved" when referring to an industrial fermentation strain means a strain having at least one desirable phenotypic modification of the progenitor strain. Illustrative of such desirable phenotype modifications include ability to grow at higher temperatures, increased growth rate upon fermentation at higher temperatures and loss of mobilization of plasmids residing within the strain.

The property of "mobilization" as used herein refers to the transmissibility of a plasmid residing within a fermentation strain.

As used herein the phrase "eliminating the cryptic plasmid from the fermentation strain" refers to the process of curing a fermentation strain of a cryptic plasmid. The present invention also encompasses modifications of the cryptic plasmid nucleic acid made through recombinant means, such as deletions, insertions, mutations, which interrupt the plasmid and its function in the host cell.

Oxidative enzymes associated with the biocatalysis of D-glucose to pathway intermediates include D-glucose dehydrogenase, D-gluconate dehydrogenase and 2-keto-D-gluconate dehydrogenase. Reductive enzymes associated with the biocatalysis of pathway intermediates into desired end-products include 2,5-diketo-D-gluconate reductase (DKGR), 2-keto reductase (2-KR) and 5-keto reductase (5-KR). Such enzymes include those produced naturally by the host strain or those introduced via recombinant means.

As used herein, the term "heterologous" refers to proteins such as enzymes that are not naturally present in host industrial fermentation strains but which have been introduced through recombinant DNA technology.

As used herein, the term fermentation refers to the range of 10 L to 500,000 L cultures.

**Description of the Preferred Embodiments**

The present invention relates to the identification and isolation of a cryptic plasmid from a strain of the family *Enterobacteriaceae*. Eliminating this plasmid from the *Enterobacteriaceae* strain reduced the mobilization properties of a resident plasmid, allowed the organism to grow at higher temperatures than the progenitor strain and provides a means for producing desirable end products in shorter fermentation runs thereby reducing the cost of their production. The following is a description of a preferred embodiment. The techniques disclosed herein are applicable to other embodiments of the present invention.

**I. Biocatalysis**

The present invention encompasses strains of the family *Enterobacteriaceae*. Preferred strains of the family *Enterobacteriaceae* are those that produce 2,5-diketo-D-gluconic acid from D-glucose solutions, including *Pantoea,* are described in Kageyama et al. (1992, International Journal of Systematic Bacteriology vol. 42, p. 203-210). The metabolic pathway of carbohydrate metabolism in recombinant 2-KLG producing strains is disclosed in Lazarus et al. (1990, Proceedings 6$^{th}$ international Symposium on Genetics of Industrial Microorganisms, Strasbourg, Vol. II 1073-1082).

Biocatalysis begins with a suitable carbon source ordinarily used by *Enterobacteriaceae* strains, such as glucose. Other metabolite sources include, but are not limited to galactose, lactose, fructose, or the enzymatic derivatives of such. In addition to an appropriate carbon source, fermentation media must contain suitable minerals, salts, cofactors, buffers and other components, known to those of skill in the art for the growth of cultures and promotion of the enzymatic pathway necessary for production of desired end-products.

In one illustrative *Pantoea* pathway, D-glucose undergoes a series of oxidative steps through enzymatic conversions, which may include the enzymes D-glucose dehydrogenase, D-gluconate dehydrogenase and 2-keto-D-gluconate dehydrogenase to give intermediates which may include, but are not limited to GA, 2KDG, and 2,5-DKG, see United States Patent 3,790,444. These intermediates undergo a series of reducing steps through enzymatic conversions, which may include the enzymes 2,5-diketo-D-gluconate reductase (DKGR), 2-keto reductase (2-KR) and 5-keto reductase (5-KR) to give end products which include but are not limited to 2KLG and IA.

The present invention also encompasses other metabolic pathways and intermediates naturally occurring in or recombinantly introduced into *Enterobacteriaceae* strains, such as for example, a pathway that proceeds through the intermediate sorbitol.

In a preferred embodiment of the present invention, the preferred fermentation strain is *Pantoea citrea*, ATCC accession number 39140. The present invention encompasses the production of desired pathway end products obtained entirely through *in vivo* methods or combined *in vivo/in vitro* methods.

**II. Recombinant Introduction of Enzymes into Fermentation Strains**

Any enzymes necessary for directing a *Enterobacteriaceae* strain carbohydrate pathway into desired end-products can be introduced via recombinant DNA techniques known to those of skill in the art if such enzymes are not naturally occurring. Alternatively, enzymes that would hinder a desired pathway can be deleted by recombinant DNA methods. The present invention includes the recombinant introduction or deletion of any enzyme or intermediate necessary to achieve a desired pathway. As used herein, recombinant DNA technology includes in vitro recombinant DNA techniques, synthetic techniques and in vivo recombinant/genetic recombination. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing "Associates and Wiley Interscience, N.Y.

In one embodiment of the present invention, nucleic acid encoding DKGR is recombinantly introduced into the *Pantoea* fermentation strain. Many species have been found to contain DKGR particularly members of the *Coryneform* group, including the genera *Corynebacterium*, *Brevibacterium*, and *Arthrobacter*.

In one preferred embodiment of the present invention, 2,5-DKGR from *Corynebacterium* sp. strain SHS752001 (Grindley et al., 1988, Applied and Environmental Microbiology 54: 1770-1775) is recombinantly introduced into a *Pantoea citrea* and the desired end product is 2KLG, a precursor to ascorbic acid. Production of recombinant 2,5

DKG reductase by Erwinia herbicola is disclosed in United States Patent No. 5,008,193 to Anderson et al.

A preferred plasmid for the recombinant introduction of non-naturally occurring enzymes or intermediates into a strain of *Enterobacteriaceae* is RSF1010, a mobilizable, but not self transmissible plasmid which has the capability to replicate in a broad range of bacterial hosts, including Gram - and Gram+ bacteria. (Frey et al., 1989, The Molecular biology of IncQ plasmids. In: Thomas (Ed.), Promiscuous Plasmids of Gram Negative Bacteria. Academic Press, London, pp. 79-94). Frey et al. (1992, Gene 113:101-106) report on three regions found to affect the mobilization properties of RSF1010. In a preferred embodiment, mobilization defective RSF1010 mutants are used for the recombinant introduction of non-naturally occurring enzymes into *Enterobacteriaceae* strains that have been cured of a cryptic plasmid.

**III. Growth Conditions**

Typically *Enterobacteriaceae* host cells are grown in the range of about 28°C to about 37°C in appropriate culture media. General growth conditions are disclosed in Truesdell et al., (1991, Journal of Bacteriology, 173: 6651-6656) and Sonoyama et al. (1982, Applied and Environmental Microbiology, vol. 43, p. 1064-1069). *Pantoea citrea* ATCC accession number 39140 has a nicotinic acid growth requirement which can be provided by nicotinomide at 100 μg/ml (Sigma). Culture media may be supplemented by the presence of selectable markers such as antibiotic resistance genes, including but not limited to tetracycline, ampicillin or chloramphenicol.

Three physical parameters which affect fermentation performance include dissolved oxygen content, pH and temperature. The rate of oxidation of metabolite sources can be limited by oxygen availability. However, the oxidation reaction will go to completion as long as oxygen is continually supplied to the culture media. In *Pantoea citrea* fermentation, air is continually provided to the fermentation tanks.

The genera *Pantoea* maintains metabolic activity under a wide range of pH conditions. Preferred pH is in the range of 4.0 to 7.5. Table 1 discloses the pH optimum for desired pathway conditions of *Pantoea citrea*.

| TABLE 1 | |
|---|---|
| Condition | pH Opt. |
| 1. Growth | 6.0 - <7.5 |
| Glucose to 2-KDG (Ox.) | 4.0-5.5 |
| 2-KDG to 2,5 DKG (Ox.) | 5.0-5.5 |
| 2,5-DKG to 2-KGA (Red.) | 5.5-6.0 |
| 2-KGA to Idonate (Red.) | 6.5-7.5 |
| Idonate to 2-KGA (Ox.) | 5.5-6.5 |

Several combinations of acid and base can be used for pH control, including but not limited to, phosphoric acid, sulfuric acid, hydrochloric acid, sodium bicarbonate, calcium carbonate, sodium hydroxide, ammonium hydroxide, and calcium hydroxide.

The optimum growth temperature of *Pantoea* citrea ATCC accession number 39140 containing the cryptic plasmid is 28 to 30°C. In ATCC accession number 39140 that has been cured of the cryptic plasmid, growth can occur at temperatures above 30°C up to about 36°C. Figures 2 and 3 illustrate the growth characteristics at 28°C, 32°C, 36°C of a pS+ strain vs a pS- strain. The growth differential between pS+ and pS- strains appears more pronounced in minimal media conditions. Accordingly, the present invention provides the unexpected advantage of providing a method for producing improved fermentation strains that can grow at higher temperatures under minimal media conditions, thereby reducing the overall cost of fermentation, i.e., production of end products. Because the kinetic parameters for various enzymes in the carbohydrate metabolism pathway may be affected by elevated temperatures, the relative ratios of end-products may be affected. Accordingly, another unexpected advantage of culturing a pS- strain under elevated temperatures is to affect a shift in the ratios of end-products.

Biocatalysis end products can be measured by HPLC analysis of the fermentation broth with standard concentrations being used as controls.

## IV. Nucleic Acid Identification Methods

Means for identifying a cryptic plasmid nucleic acid within a *Enterobacteriaceae* species include hybridization screening techniques that use radioactive or enzymatically labeled probes to screen the suspect species nucleic acid under high stringency conditions. The term probe refers to a portion, fragment or segment of SEQ ID NO:1 or SEQ ID NO:2 that is capable of being hybridized to a desired target nucleotide sequence and probes can be used to detect, amplify or quantify nucleic acid. Probes may be labeled by nick translation, Klenow fill-in reaction, PCR or other methods well known in the art.

Alternatively, polymerase chain reaction (PCR) based strategy (United States Patents 4,683,195; 4,800,195; 4,965,188) may be used to identify nucleic acid of the cryptic plasmid. Oligonucleotides preferably in the range of 18-22 nucleotides derived from SEQ ID NO:1 or SEQ ID NO:2 serve as primers for the PCR reaction with the template comprising nucleic acid derived from suspect *Pantoea* species. Any PCR products or other identified nucleic acid may be subcloned and subjected to nucleic acid sequencing to confirm the identity of the cryptic plasmid.

Methods for nucleic acid sequencing are well known in the art. Conventional enzymatic methods employ DNA polymerase Klenow fragment, SEQUENASE® (US Biochemical Corp, Cleveland, OH) or Taq polymerase to extend DNA chains from an oligonucleotide primer annealed to the DNA template of interest.

The cryptic plasmid pS was sequenced in two parts and is shown in SEQ ID NO:1 and SEQ ID NO:2. Scientific programs such as, DNASTAR (DNASTAR Inc., 1228 South Park St., Madison, WI 53715) are available to those of skill in the art for joining the two sequences, SEQ ID NO:1 and SEQ ID NO:2, in order to obtain a continuous sequence.

## V. Methods for Curing *Enterobacteriaceae* Strains of the Cryptic Plasmid

Methods for curing the cryptic plasmid from *Enterobacteriaceae* are described in Jeffrey Miller (1972, in Curing of Episomes from E.Coli strains with Acridine Orange from Experiments in Molecular Genetics, Cold Spring Harbor Laboratories, pg. 140). In this method, acridine orange is added to 5 ml cultures of an *Enterobacteriaceae* strain at 125 µg/ml and allowed to grow overnight at 37 °C. The following day, the cultures are plated out and individual colonies are used to prepare plasmid nucleic acid. The nucleic acid is analysed by means known to those of skill in the art to determine the presence or absence of the plasmid.

The manner and method of carrying out the present invention may be more fully understood by those of skill in the art by reference to the following examples, which examples are not intended in any manner to limit the scope of the present invention or of the claims directed thereto.

## EXAMPLES

### I. Identification of the Cryptic Plasmid.

This example describes the initial discovery and identification of pS in Pantoea citrea. pS was discovered during a plasmid purification experiment specifically designed to find any cryptic plasmids native to the Pantoea citrea.

Pantoea citrea ATCC accession number 39140 was subjected to a plasmid preparation by standard means. Plasmid DNA was subjected to 1% agarose gel electrophoresis and a plasmid of 3.8kb, designated pS, was identified. The 3.8 kb band was excised and the nucleic acid was electroeluted and purified by precipitation. pS nucleic acid was subjected to restriction analysis via restriction endonucleases and sequenced by standard dideoxy sequencing methodology. The nucleic acid sequence of pS is shown in two halves, SEQ ID NO:1 and SEQ ID NO:2. SEQ ID NO:1 and SEQ ID NO:2 were subjected to a BLAST search (FastA Genetics Computer Group, Inc., University Research Park, 575 Science Dr. Ste. B, Madison, WI 53711) which revealed no homology to known nucleic acids. The amino acid sequence encoding by the largest open reading frame is shown in Figures 1A - 1C and is designated SEQ ID NO:3.

II. **Curing *Pantoea citrea* of pS.**

This example describes the method for curing *Pantoea citrea* of pS.

Acridine orange was added to 5 ml cultures of Pantoea citrea ATCC accession number 39140 at 125 µg/ml and allowed to grow overnight at 37 °C. The following day, the cultures were plated out and individual colonies were used to prepare plasmid nucleic acid. The nucleic acid thus prepared was analysed on a 1% agarose gel to determine the presence or absence of pS. One colony was determined to no longer contain pS. Subsequent purification of the colony and repeated efforts to isolate the plasmid DNA confirmed that this particular isolate had lost the cryptic plasmid. This culture, designated as Pantoea citrea pS- or "pS-" was used for subsequent experimentation in comparisons with Pantoea citrea containing pS or "pS+"

**III. Transmissibility of Vector Plasmids from *Pantoea citrea* to other Microbial Hosts.**

The purpose of this example was to determine the transfer frequency of expression vectors from *Pantoea citrea* to *Escherichia.coli* and *Pseudomonas aeruginosa*. Transfer frequency was assayed by mating strains of *Pantoea citrea* with *E.coli* and *P.aeruginosa* under selective conditions and in the presence of a counter selective agent or condition.

An expression vector containing nucleic acid encoding a DGKR was created using plasmid RSF1010 which was modified by deleting a region of the plasmid involved in mobilization (Frey et al., *supra*). Additionally, the cryptic plasmid, was cured from *Pantoea citrea* by the method disclosed in Example II.

**Materials and Methods**

E.coli strain 294 (endA, hsdR, $Thi^-$, $Pro^-$, $Str^S$) was used as the recipient in all the experiments pertaining to E.coli. Selective conditions included Tetracycline at 20 and 100 µg/ml and Streptomycin at 100 and 500 µg/ml. Counter selective agents or conditions include growth at 42 °C. or the presence of Irgasan at 12 µg/ml. The selective temperature conditions were chosen to be 37°C, however, it was determined that the two *Pantoea citrea* strains having been cured of the cryptic plasmid were able to grow at 37°C. the *Pantoea citrea* strain that contained the cryptic plasmid (pS-) was still unable to grow at 37°C. Accordingly, all *P. citrea* strains were tested for growth at 42°C. All three *P. citrea* strains failed to grow at this temperature under the conditions used for selection, while the recipient *E. coli* strain was able to grow at this temperature. The same conditions were used for *P. aeruginosa* as used for *E. coli*. Table II illustrates the selective and counterselective conditions used for experimentation.

TABLE 2. SELECTIVE AND COUNTERSELECTIVE AGENTS AND CONDITIONS

| MATING | SELECTIVE ANTIBIOTIC | COUNTER SELECTIVE AGENT OR CONDITION |
|---|---|---|
| *P. citrea* -pS (pD92) X *E. coli* 294 | Tetracycline 20 µg/ml | Growth @ 42°C |
| *P. citrea* -139-2A (Δ18) X *E. coli* 294 | Streptomycin 100 µg/ml | Growth @ 42°C |
| *P. citrea* -pS (Δ18) X *E. coli* 294 | Streptomycin 100 µg/ml | Growth @ 42°C |
| *P. citrea* -pS (pD92) X *P. aeruginosa* PA01 | Tetracycline 100 µg/ml | Irgasan 12 µg/ml |
| *P. citrea* -139-2A (Δ18) X *P. aeruginosa* PA01 | Streptomycin 500 µg/ml | Irgasan 12 µg/ml |
| *P. citrea* -pS (Δ18) X *P. aeruginosa* PA01 | Streptomycin 500 µg/ml | Irgasan 12 µg/ml |

**Mating**

Each strain of *Pantoea citrea* was mated with E.coli 294 and P. *aeruginosa* PA01 on three separate occasions. As a negative control, each strain of P. *citrea* was used in a mock mating without a recipient. No growth was observed on any of the selective plates in these mock matings.

The frequency of transfer was calculated as follows:

$$\text{Frequency of transfer (\%)} = \frac{\text{\# Transconjugants/ml}}{\text{\# Input Donor/ml}} \times 100$$

The data from all matings are shown in Table 3.

TABLE 3. FREQUENCY OF TRANSMISSION OF VECTOR PLASMIDS TO *E. COLI* 294 AND *P. AERUGINOSA* PA01

| Mating | Exp. 1 | Exp. 2 | Exp. 3 | Mean |
|---|---|---|---|---|
| *P. citrea* -pS (pD92) X *E. coli* 294 | .001% | .0003% | .0008% | .0007% |
| *P. citrea* -139-2A (Δ18) X *E. coli* 294 | .0017% | .0002% | .0005% | .0008% |
| *P. citrea* -pS (Δ18) X *E. coli* 294 | .0009% | .0002% | .00007% | .0004% |
| *P. citrea* -pS (pD92) X *P. aeruginosa* PA01 | .0006% | .0001% | .00003% | .0002% |
| *P. citrea* -139-2A (Δ18) X *P. aeruginosa* PA01 | .0001% | .0003% | .0001% | .0002% |
| *P. citrea* -pS (Δ18) X *P. aeruginosa* PA01 | .0003% | .00015% | .0001% | .0002% |

**Analysis of Transconjugants**

Because of the very low apparent frequency of transmission that was observed genetically, it was necessary to examine transconjugants for the presence of vector plasmids to determine whether transmission had actually occurred or whether the colonies observed on the selection plates were due to selection of chromosomal mutations that result in resistance to the antibiotics used. 10 transconjugants from each mating were examined for the presence of plasmids. Plasmids were detected in all cases.

**Results**

The results shown in Table 3 indicate that transmission of the expression vector from *P. citrea* cured of the small cryptic plasmid (pS-) to *E. coli* and P. *aeruginosa* has been reduced about 1000 fold in comparison to *P. citrea* having the small cryptic plasmid present.

Transmission of RSF1010 based expression vector having the deletion disclosed in Frey et al., supra, to E *coli* and P. *aeruginosa* from P. *citrea was* reduced by about 1000 fold in comparison to transmission of the RSF1010 plasmid without the mutation to these organisms from P. *citrea.*

Various other examples and modifications of the foregoing description and examples will be apparent to a person skilled in the art after reading the disclosure without departing from the spirit and scope of the invention, and it is intended that all such examples or modifications be included within the scope of the appended claims. All publications and patents referenced herein are hereby incorporated in their entirety.